## REMARKS

Reconsideration and withdrawal of the claim rejections are requested in view of the amendments and remarks herein.

## I. STATUS OF CLAIMS AND FORMAL MATTERS

Claims 1-5, 8-12, 14-16 and 27-29 are pending in this application. Claims 1, 8, 12, 14-16 and 27 are amended; claims 6, 7, 13 and 17-26 are cancelled; and claims 28 and 29 are added. Support for the hybridization conditions recited in claims 27 and 28 can be found on page 5, lines 27-37, of the specification. The remaining amendments place the claims in better form, and do not affect the scope. No new matter is added.

It is submitted that the claims, herewith and as originally presented, are patentably distinct over the prior art cited by the Examiner, and that these claims are and were in full compliance with the requirements of 35 U.S.C. §112. The amendments of the claims, as presented herein, are not made for purposes of patentability within the meaning of 35 U.S.C. §§§§ 101, 102, 103 or 112. Rather, these amendments are made simply for clarification and to round out the scope of protection to which Applicants are entitled. Furthermore, it is explicitly stated that the herewith amendments should not give rise to any estoppel, as the herewith amendments are not narrowing amendments.

## II. THE REJECTIONS UNDER 35 U.S.C. §112, 2ND PARAGRAPH ARE OVERCOME

Claims 1-6, 8-12, 14-16 and 27 were rejected under 35 U.S.C. §112, second paragraph, as allegedly being indefinite. The rejections are traversed.

The Office Action objected to the phrase "under stringent conditions" in claims 1, 6 and 27. Claim 6 has been cancelled, and claims 1 and 27 no longer recite the phrase. The recitation of specific hybridization conditions has been added to claim 27.

The phrase "wherein a cosuppression effect is achieved" in claim 10 has been deemed indefinite. It is submitted that "cosuppression" is a standard term whose meaning is well known in the transgenic plant art. As evidence of this assertion, several review articles on cosuppression in plants (Jorgensen, *Trends Biotechnol.* 8:340-4; 1990; Flavell *et al.*, *Curr. Top. Microbiol. Immunol.* 197:43-56; 1995; Smyth, *Curr. Biol.* 7:R793-5; 1997), published before the earliest priority date of this application, are enclosed. Note that Jorgensen and Flavell *et al.* are cited on page 15, lines 1-3, of the specification. Cosuppression is not "obtained from two different regulatory elements or from regulatory elements and other source", as suggested by the

Office Action. Rather, it occurs in a cell when the presence of one gene (such as SEQ ID NO:1) suppresses the activity of an endogenous, related gene (such as native wheat starch synthase). This effect is well known and understood in the art, as is demonstrated by the enclosed review articles. See, in particular, the last paragraph on page 340 of Jorgensen, the paragraph bridging pages 43 and 44 of Flavell *et al.* and the second paragraph of Smyth, all of which demonstrate a common understanding of the meaning of the term cosuppression, as it is used in the art. Therefore, the term "cosuppression" is not indefinite, and would not be so interpreted by the skilled artisan.

Reconsideration and withdrawal of the rejections under §112, second paragraph, are requested.

## III. THE REJECTIONS UNDER 35 U.S.C. §112, 1ST PARAGRAPH ARE OVERCOME

Claims 1-6, 8-12 and 14-16 were rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking adequate written description. The rejection is traversed.

Hybridization language is no longer recited in claim 1, obviating this aspect of the rejection. Therefore, the remaining discussion with respect to the written description rejection will center around claim 27. To that end, specific hybridization conditions have been added to part (c) of claim 27, to more clearly define how hybridization is to be performed.

The Examiner's attention is directed to Example 9 of the U.S.P.T.O.'s "Synopsis of Application of Written Description Guidelines". The fact pattern presented in Example 9 is analogous to the current situation: a DNA sequence has been identified and specific hybridization conditions are stated. The following elements are required by Example 9: 1) hybridization techniques using a known DNA sequence as a probe, under the recited conditions, were conventional in the art at the time of filing; 2) the claim (in this case, new claim 28 and part (c) of claim 27) is drawn to a genus of nucleic acids, all of which must hybridize with the disclosed species (in this case, nucleotides 9-570 of SEQ ID NO:1 or a ribonucleotide sequence corresponding therewith) and must encode a protein with a specific activity (*i.e.* the function of a wheat starch synthase); 3) the claimed species, SEQ ID NO:1, is novel and unobvious; 4) the single species disclosed, SEQ ID NO:1, is within the scope of the claimed genus; 5) there is actual reduction to practice of the disclosed species. The instant claims and application meet all of these requirements; therefore, written description is present.

Turning to page 4 of the Office Action, it is stated that the scope of the claims includes various known, and unknown and unidentified genes that either encode or do not encode a polypeptide. This is incorrect. Claim 27 requires that the nucleic acid molecule encode a protein of a specific function. If a nucleic acid molecule comprises nucleotides 9-570 of SEQ ID NO:1, and encodes a protein with the function of a wheat starch synthase, it falls within the claims. Just because every species of a genus has not been identified does not preclude a claim to the genus.

The Office Action goes on to say that the structures of the claimed nucleic acid molecules have not been disclosed, that "there is no known or disclosed correlation between function and structure of the non-described regulatory elements and untranslated regions of the gene", and finally, that "[t]he specification also fails to provide the structural features of the nucleic acid molecule encoding a protein have a wheat starch synthase activity". This is also inaccurate. Firstly, the structure of a nucleic acid molecule encoding a wheat starch synthase has been provided as SEQ ID NO:1. The skilled artisan would not expect substantial variation among species encompassed within the scope of the claims, because the stringent hybridization conditions set forth in claims 27 and 28 yield structurally similar DNAs to SEQ ID NO:1. Further, nucleotides 9-570 of SEQ ID NO:1 are not restricted to "regulatory elements and untranslated regions of the gene", as is suggested by the Office Action. The coding sequence of SEQ ID NO:1 begins at position 280; therefore, a substantial portion of nucleotides 9-570 correspond with the N-terminal end of the protein.

Limiting the Applicants to only a nucleic acid molecule of SEQ ID NO:1 or a nucleic acid molecule that encodes SEQ ID NO:2 would unfairly narrow the scope of the invention. For example, other parties could use nucleic acid molecules distinct from SEQ ID NO:2 that encode a structurally and/or functionally identical enzyme, to practice this very invention, and they would fall outside the literal scope of the claims. Such a consequence is obviously contrary to the intended function of the patenting system.

Claims 1-6, 8-12 and 14-16 were rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement. The rejection is traversed.

The claims involve a genus of nucleic acid molecules having structural and functional similarity to SEQ ID NO:1. These features are required by the claims. Further, specific guidance is given in the specification regarding how to isolate a nucleic acid molecule that hybridizes to a portion of the claimed nucleic acid molecules. Therefore, the structural

characteristics of the claimed nucleic acid molecules are clearly set forth. In addition, the claims contain the functional limitation that the nucleic acid molecule has the function of a wheat starch synthase. Characteristics of starch synthases and how to identify them are described on page 7, lines 22-29. There is no reason to expect that one of skill in the art could not identify a member of the claimed genus based on its structural and functional characteristics, and the Examiner has not provided any evidence to the contrary.

Applicants reiterate that there would be no undue experimentation on the part of the skilled artisan to isolate the claimed nucleic acid molecules. As stated by the Court of Appeals for the Federal Circuit in the case of *In re Wands*, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988):

> Enablement is not precluded by the necessity for some experimentation such as routine screening. However, experimentation needed to practice the invention must not be undue experimentation. **The key word is undue, not experimentation.** The determination of what constitutes undue experimentation in a given case requires the application of standard of reasonableness, having due regard for the nature of the invention and the state of the art. The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed ... [Emphasis added. Citations omitted].

*Id.* at 1404.

Against this background, determining whether undue experimentation is required to practice a claimed invention turns on weighing many factors summarized in *In re Wands* (*Id.*), for example, (1) the quantity of experimentation necessary; (2) the amount of direction or guidance presented; (3) the presence or absence of working examples of the invention; (4) the nature of the invention; (5) the state of the prior art; (6) the relative skill of those in the art; (7) the predictability or unpredictability of the art; and (8) the breadth of the claims.

Applying *Wands* to the instant facts, enablement is shown to exist. The fact that some experimentation may be required does not mean that it is undue. In the instant case, the specification provides considerable direction and guidance on how to practice the claimed invention. Working examples are present, all of the methods needed to practice the invention were well known, and there was a high level of skill in the art in the art at the time of filing. In order to practice the invention claimed in claim 28, for example, the skilled artisan would first need to start with a nucleic acid molecule comprising nucleotides 9-570 of SEQ ID NO:1. The

total length and sequence of the molecule are not important, provided that at least nucleotides 9-570 of SEQ ID NO:1 are contained within the molecule. It is well within the abilities of the artisan to know whether the starting material contains a specific sequence. Next, hybridization, a routine procedure in the art, is carried out, under specified conditions, to yield some number of nucleic acid molecules that hybridize to the starting material. Finally, the artisan determines whether the isolated nucleic acid molecules, or their complements, have the function of a wheat starch synthase. Guidance on how to identify starch synthases is provided on page 7, lines 22-29, of the specification. There is no part of this process that involves undue experimentation by one of skill in the art, and if the Examiner maintains the contrary opinion, it is requested that he specifically identify which aspect involves undue experimentation, and what that experimentation would be.

It is submitted that the claims are in compliance with the first paragraph of §112, and reconsideration and withdrawal of the rejections thereunder are requested.

## IV. THE REJECTIONS UNDER 35 U.S.C. §102 ARE OVERCOME

Claims 1-3, 5, 6 and 27 were rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Block *et al.*, 1996. The rejection is traversed.

As was stated in the Amendment filed on May 6, 2003, Block *et al.* describes only a fragment of the claimed molecule, whereas the instant application discloses the full-length clone for the first time. There is no evidence that the nucleic acid sequence of Block *et al.* encodes a protein with the function of a wheat starch synthase. In addition, a claim is anticipated only if each and every element set forth in the claim is found in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). Claim 1, and dependent claims, are drawn to nucleic acid molecules having the sequence of SEQ ID NO:1 or encoding SEQ ID NO:2, with the function of a wheat starch synthase. Block *et al.* clearly cannot anticipate claim 1, as it discloses neither of these sequences, nor does it teach that the disclosed sequence has any function at all. The Office Action states, on page 8, that "Block teaches a wheat Triticum aestivum soluble starch synthase mRNA sequence, ... which is 100% identical to base [sic] 718-2771 of SEQ ID No. 1. Since it is a wheat soluble starch synthase mRNA sequence, the protein encoded by said sequence would have wheat starch synthase activity." As was pointed out above, the coding sequence of SEQ ID NO:1 begins at nucleotide 280; therefore, there are 438 coding nucleotides missing from the sequence of Block *et al.*, which

translates to 146 amino acids of SEQ ID NO:2 that are not present in the molecule of Block *et al.* Given that a substantial portion of the N-terminus is missing from the protein, there is no evidence at all that it would have any enzymatic function whatsoever.

Furthermore, because the sequence of Block *et al.* does not contain nucleotides 9-570 of SEQ ID NO:1, it will not hybridize with those nucleotides, as recited in claim 28.

Claims 1-6, 8, 9, 12, 14-16 and 27 were rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Block *et al.*, 1997. The rejection is traversed.

As discussed in the Amendment filed on May 6, 2003, Block *et al.* relates to soluble starch synthase II (SSII), which is an entirely different enzyme than that of the instant invention, SSI. The sequence of SSII is different from that of SEQ ID NO:2, and the nucleic acid molecule of Block *et al.* will not hybridize, as claimed, to the claimed nucleic acid molecules. Furthermore, Applicants restate their position above that there is no evidence in the cited reference, nor has the Examiner provided any other evidence, that the molecule of Block *et al.* has any biological function. This molecule is missing the first 85 amino acids of the protein of the instant invention. In addition, base 533 of SEQ ID NO:1 is in the middle of a codon, meaning that if one started translating at the first nucleotide of Block *et al.*, it would not even be in frame with the coding sequence of SEQ ID NO:1. The artisan is not taught by Block *et al.* where to start translating.

It is submitted that neither cited Block *et al.* references anticipates the pending claims. Subsequently, reconsideration and withdrawal of the §102 rejections are requested.

## CONCLUSION

Applicants believe that the application is in condition for allowance, and favorable reconsideration of the application and prompt issuance of a Notice of Allowance are earnestly solicited. Alternatively, consideration and entry of this paper is requested, as it places this application into better condition for purposes of appeal.

Respectfully submitted,

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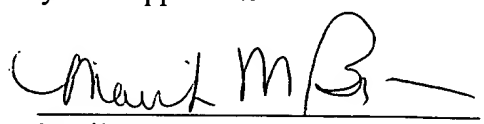
By: 

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# Trends in BIOTECHNOLOGY

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while others examined methodologies to design fully integrated processes. The consensus opinion was that integration of processes is the direction to take, though there were diverse views as to how best to achieve this goal. Combining multiple functions into a single operation has the benefit of reducing the number of steps and increasing the overall yield, but does not necessarily lead to process robustness. When two steps are used in sequence, for example, if the first is variable in performance, then the second can be designed to remove impurities or contaminants that flow through the first. If a single-unit operation is used then this possibility is lost. Application of computer-aided design techniques works well for cost minimalization but can not predict trace impurities or contaminants that determine product quality because analytical descriptions are, as yet, too imprecise.

**Product finishing**

An important area of downstream processing is product finishing. This topic is neglected in most conferences but was nicely addressed on this occasion. C. R. Hill (Celltech Ltd, UK) reviewed the problems associated with product finishing and pointed out that impurities and contaminants can be introduced into the process from raw materials, host-cell synthesis, and in-process reactive conditions. Attention to detail in operation and design, development of sensitive analytical methods, and establishing rigorous quality controls are essential to the success of any manufacturing process for therapeutic materials. These points were amplified by the other speakers in an entire session devoted to this important topic.

**The search for purity**

This conference provided an excellent opportunity for researchers and practitioners to come together and discuss work on separations for biotechnology. While advancements were cited on all fronts, it is disappointing that little attention was given by the individual researchers to the problems of removing low-level impurities and contaminants such as viruses, nucleic acids and isomeric or modified forms of proteins. The need to reach levels of 99.999% purity, or better, is the most difficult challenge today in protein purification for therapeutic use; for some reason researchers seem unwilling or unable to accept this challenge. Perhaps we can look forward to advances in this area, too, in the next of this series of conferences on Separations for Biotechnology.

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# UNDERCURRENTS

# Altered gene expression in plants due to *trans* interactions between homologous genes

Two recent publications[1,2] on the genetic engineering of flower color have reported the intriguing observation that ectopic transgenes can participate in a homology-based interaction with endogenous plant genes, resulting in suppression of the expression of both the ectopic gene and the endogenous gene. Better understanding of this phenomenon may eventually shed some light on the dynamic behavior of genes in the nucleus. Furthermore, the phenomenon is likely to have significant practical applications in the manipulation of plant phenotypes for use in agriculture and the plant sciences.

**Co-suppression of anthocyanin biosynthetic enzymes in petunia**

In many plants, flower color is determined by the production of anthocyanin pigments. In an attempt to overproduce flower pigments in petunias, a chimeric gene encoding chalcone synthase (CHS), a key enzyme in the anthocyanin pigment biosynthetic pathway (Fig. 1), was introduced; the results, however, were surprising in several ways.

First, the introduction of a CHS transgene to ectopic sites in the petunia genome was found to suppress the expression of both alleles of the endogenous CHS gene *in trans*, causing the production of pure white or patterned flowers[1] (see Box 1). Similar results were obtained independently with CHS, and then also with another petunia anthocyanin gene encoding the enzyme dihydroflavonol-4-reductase (DFR) (Fig. 1)[2]. It was demonstrated that in both cases only homologous gene expression was suppressed, not that of other genes in the pathway[1,2].

However, the really surprising result was that suppression of the endogenous gene was not simply the result of transgene expression. This could be shown because the suppression phenomenon was somatically reversible in both CHS and DFR transformants (i.e. white flowers sometimes reverted to wild-type purple flowers) (see Box 1). In purple revertant flowers the expression levels of both the ectopic transgene and the endogenous homologous gene were found to increase coordinately and substantially (50-fold) restoring expression of the endogenous gene to normal levels[1,2]. This indicated that both genes must be coordinately suppressed in the white flowers, suggesting the provisional term 'co-suppression' for this phenomenon[1].

In this article, we explore the possible mechanisms and significance of co-suppression through comparison of the co-suppression phenomenon in *Petunia hybrida* with other studies on *trans* interactions in plants and fungi and on epigenetic phenomena in plants. The most salient features of co-suppression are shown in Box 2.

### Other *trans* interactions in transgenic plants

Results from other recent studies suggest that co-suppression of the expression of duplicated gene sequences could be a widespread phenomenon in plants and not limited to petunia CHS and DFR genes. At a recent UCLA Symposium (Keystone, CO, USA, April 1990) on Molecular Strategies for Crop Improvement, reversible suppression of an endogenous potato starch synthase gene following the introduction of a functional copy of the gene was reported; suppression of the ectopic gene was not examined, only implied by the loss of phenotype[3]. Working with a chimeric β-glucuronidase (GUS) transgene in tobacco, Hobbs and co-workers found low GUS expression in transformants with two GUS genes, but high GUS expression in progeny where the two GUS genes had segregated away from each other, suggesting co-suppression of GUS genes in the original transformant (Hobbs, pers. commun.). Interestingly, low expression was correlated with methylation of the DNA. These results also seem to suggest that co-suppression may not depend on the duplicated gene being of plant origin.

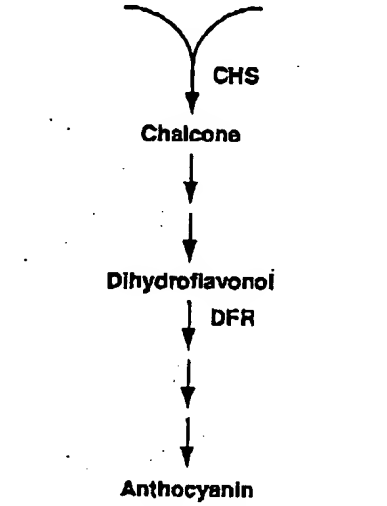
In several other examples, suppression of a homologous gene has been observed following the introduction of a partial copy of an active gene, e.g. firefly luciferase in tobacco (Meeks-Wagner, pers. commun.), bacterial nopaline synthase in tobacco (Rothstein, pers. commun.) and tomato polygalactonurase in tomato (Grierson, pers. commun.). In these examples, expression of the partial gene copy was not examined, nor was reversibility tested.

Epigenetic variation in the expression of maize DFR transgenes in petunia may also be the result of co-suppression. Petunia plants carrying a single maize DFR transgene usually exhibit uniform expression of the transgene, whereas plants that exhibit erratic or no expression of the transgene usually carry multiple copies of that gene[4]. Methylation of transgenes was strongly correlated with lack of uniform expression. Exceptions, in which single copy transgenes are methylated and not uniformly expressed, indicate either that, even if sequence duplication promotes suppression, it cannot be the only determinant of suppression, or that the heterologous maize DFR transgene is sometimes capable of interacting with the petunia DFR gene.

Matzke and co-workers[5,6] repeatedly observed the reversible inactivation of bacterial nopaline synthase (NOS) and kanamycin resistance (KAN) genes carried by T-DNA (T-DNA-I) on introduction of a second T-DNA (T-DNA-II). T-DNA-II carried part of a NOS gene and other sequences homologous to T-DNA-I. ('T-DNA' refers to 'transferred DNA', i.e. the segment of DNA transferred by *Agrobacterium tumefaciens* to the plant genome.) The phenotypes of the genes carried by T-DNA-II were not suppressed, i.e. suppressi n was not observed to be co-ordinate, alth ugh gene expression levels were not quantitated. Lack of expression of T-DNA-I was correlated with methylation of the DNA and occurred regardless of whether T-DNA-I was in homozyg us or hemizygous condition. Suppression of T-DNA-I was always reversed in progeny that lack T-DNA-II, but methylation was reversed only in a fraction of the cells of progeny plants[5,6]. Furthermore, in some transformants with two unlinked copies of T-DNA-II, progeny tests demonstrated that T-DNA-I was suppressed in the presence of one of two T-DNA-II loci, but not the other, suggesting that perhaps the relative locations of the two T-DNA loci play a role in determining their interaction[6].

In another study, transgenic *Arabidopsis thaliana* plants which failed to transmit their hygromycin resistance phenotype (HPT) to progeny even though the HPT gene was transmitted normally were found invariably to carry multiple copies of the HPT gene[7]. In addition, the loss of phenotype was somatically reversible, although no *trans* interactions have been demonstrated yet.

**Fig. 1**




*Schematic representation of the anthocyanin pigment biosynthetic pathway showing where the key enzymes CHS (chalcone synthase) and DFR (dihydroflavonol-4-reductase) function.*

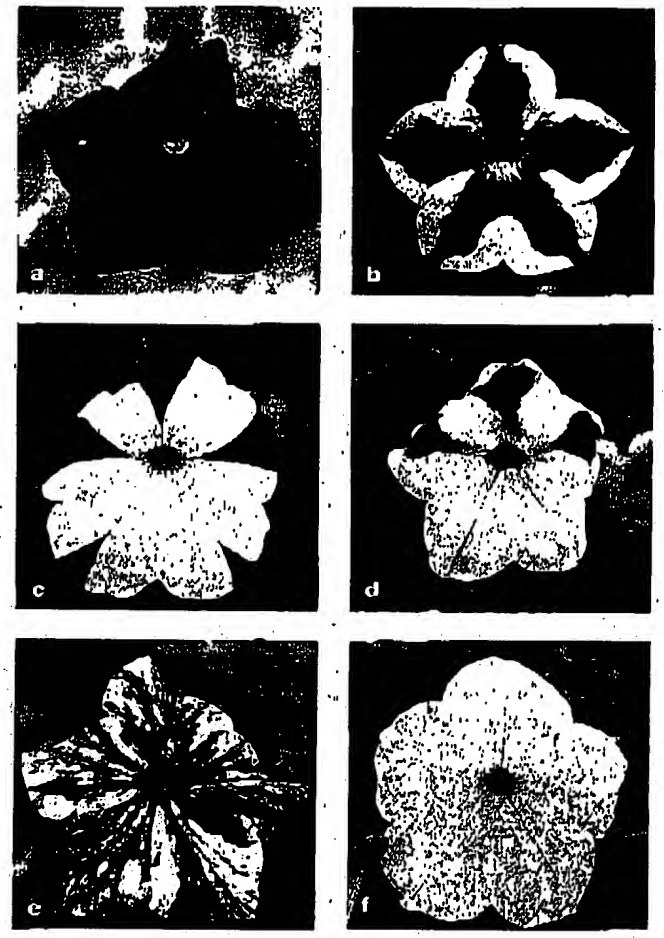
### *trans* interactions in fungi and comparisons with co-suppression

A phenomenon which parallels co-suppression has been known in fungi for several years. With few exceptions, duplicated genes in *Neurospora* are subject to a process causing irreversible inactivation of both gene copies at a pre-meiotic stage of the sexual phase[10]. This 'pre-meiotic inactivation' of duplicated sequences is associated with extraordinarily high rates of point mutation and cytosine methylati n and, accordingly, was termed RIP, for repeat-induced point mutation[11]. Recently, work in another filamentous fungus, *Ascobolus immersus*, has also shown that duplication of genes leads to their premeiotic inactivation[12,13]. However, in *Ascobolus*, gene inactivation appears to be reversible, and no sequence alterations have yet been reported. Of great significance mechanistically, it was found that, in both fungi, inactivation of the duplicated genes is coordinate, i.e. individual members of a duplicate pair are never inactivated alone[13–15]. Inactivation occurs whether the sequence is duplicated in tandem or in two unlinked locations[10,13,15]. Thus, pre-meiotic inactivation appears to be the result of

Box 1

## Co-suppr ssion in p tunias is under precise dev lopm ntal c ntrol

An advantage of using pigment genes for the study of *trans* interaction is the ability to observe easily spatial patterns of gene expression. Co-suppression of flower pigment genes was found to cause strikingly beautiful, highly regular patterns that were relatively stable, as well as to cause erratic, less stable patterns[1,2] (see photos a–f). Stable patterns were always non-clonal; less stable patterns were usually non-clonal. Furthermore, analysis of somatic reversion events (where flower color changes from pure white to solid purple) demonstrated that the CHS genes reverted in both epidermal and mesophyll cell layers (which are not clonally related) to their normal non-suppressed state[6]. Thus, reversion appears to occur in a co-operative fashion among adjacent cells, i.e. cell-to-cell communication determines whether a branch gives rise to purple or white flowers and also whether individual cells are pigmented in patterned flowers. Reversion is abrupt and almost always occurs at branch points, suggesting that perhaps the primary event in reversion occurs in an axillary meristem[6]. Once determined, suppressed and active states are relatively stable, though still reversible, at a frequency that seems to vary among transformants. Based on these observations it is apparent that co-suppression can be subject to precise developmental control, but also to certain stochastic processes. Thus, co-suppression is an epigenetic phenomenon that can be compared with the phenomenon of cycling of transposon activity in maize, especially as seen with the *Spm* transposable element[9].




*Flower color patterns produced by introduction of CHS transgene. Each flower shown was taken from a different transformant. Variability among flowers of the same plant is discussed in Box 2 and Ref. 1. Isogenic progeny populations of some transformants can produce nearly the full range of patterns seen here. This suggests that epigenetic factors determine pattern variability. Flowers from control transformants without the CHS transgene were all uniformly pigmented. All white regions in the flower patterns shown, including the small white spot in (a), are caused by the CHS transgene. The pattern of flower (b) is known as the 'Cossack dancer'.*

an interaction, either direct or indirect, between homologous sequences, resulting in the detection of the duplication by a relatively efficient process[11,12,15].

There is a potentially important difference between co-suppression in a plant cell and pre-meiotic inactivation in a fungal cell: whereas co-suppression may be observed readily in somatic, diploid cells that are not necessarily part of the germ line, pre-meiotic inactivation occurs mainly in germ-line cells. However, pre-meiotic inactivation and co-suppression are similar in that both can occur at high frequency, even when the duplicated sequences are unlinked (it was suggested that such a phenomenon could not be tolerated in a diploid nucleus such as in a somatic cell of a higher organism)[14].

Unlike the *Neurospora* genome, which carries essentially no repetitive DNA sequences other than ribosomal RNA genes, the genomes of higher plants carry huge numbers of repetitive sequences (often highly methylated), and large numbers of active multigene families. The multiploid nature of plants indicates that the normal interaction between such homologs differs in some way from *trans* interactions that occur in co-suppression. The abundance of repetitive DNA sequences in plants places serious constraints on the mechanism of any process which suppresses the expression of duplicated genes. This suggests that the process of co-suppression in plants must be discriminat ry in some way.

### The mechanism of co-suppression?

Co-suppression is a *trans* interaction between duplicated genes that is dependent on homology (see Box 2). However, in transgenic plants gene duplication does not always lead to suppression. Thus, it is clear that homology alone is not sufficient for co-suppression to occur. This suggests a class of hypotheses in which the 'susceptibility' or 'resistance' of homologous genes to co-suppression is determined by the relative positions of the homologous genes in the genome and/or by the sequence context of the two homologous genes. These hypotheses invoke close proximity or special relationships between chromosome regions carrying the genes, or more localized effects of neighboring DNA sequences on the potential for the *trans* interaction. Hypotheses invoking these factors would predict that altering the sequence context or location of a gene in the appropriate manner would alter its resistance to co-suppression. A corollary of such hypotheses would be that essential

Box 2

## Important features of co-suppression

The introduction of either a CHS (chalcone synthase) or a DFR (dihydroflavonol-4-reductase) transgene to an ectopic position in the petunia genome can result in the co-ordinate and reversible suppression (co-suppression) of the transgene and both alleles of a homologous, endogenous gene, but apparently no other gene. Suppression is always reversed in progeny that lack the transgene. Intriguingly, co-suppression of CHS and DFR genes in petunia occurs even when the homology between the ectopic transgene and the endogenous gene is limited to the coding sequences of these genes, as well as when they are driven by promoters unrelated to those of the endogenous genes[1,2]. In addition, the effect is specific to the homologous gene pair, i.e. no other related, non-homologous genes from the same biochemical pathway are effected. Furthermore, duplicated sequences that are of other than plant origin (e.g. GUS) also appear to be subject to co-suppression. It is significant, too, that no suppression effect is observed in half or more of transgenic plants, and that the activity of the duplicated gene pair can be in either a suppressed state or a non-suppressed state in different branches of the same plant, and in isogenic sibling progeny[1,2,5,8] (see Box 1). Thus, suppression is not obligatory in the presence of a gene duplication. It seems likely that suppression is determined to some extent by the relative locations of the interacting sequences[6], and that both developmental and stochastic processes can trigger suppression.

Together, these observations strongly suggest that co-suppression is not simply a dosage effect resulting from competition between binding sites for transcription factors; nor is it the result of a system that detects specific duplicated plant genes. Rather, co-suppression seems to be the result of a homology-dependent interaction between any or most homologous sequences under appropriate conditions.

gene families, such as ribosomal RNA genes, have evolved sequence contexts or locations that are relatively 'immune' to co-suppression. A different hypothesis is that the nature of the homologous sequence itself, perhaps even a common but specific site, determines its susceptibility, or resistance, to co-suppression. Similar hypotheses have been discussed in relation to pre-meiotic inactivation[16].

The actual mechanism behind co-suppression might be either a direct interaction via ectopic pairing of the duplicated sequences, or an indirect one, perhaps via transcripts[1,2]. Ectopic pairing could easily be imagined to be dependent on sequence context, while an interaction between a diffusible RNA and a homologous gene might be distance dependent. An intermediate possibility is direct ectopic pairing via transcription complexes. Clearly, any proposed mechanism must be able to account for the suppression of *both* alleles of an endogenous gene in the presence of the ectopic gene.

### A comparison of co-suppression with other epigenetic phenomena in plants

The reversible nature of co-suppression and the non-clonal nature of the flower color patterns associated with it (see Box 1) are reminiscent of the phenomena of paramutation and epimutation in plants. Paramutation is an inter-allelic *trans* interaction, where one allele (paramutagenic) causes directed, heritable and reversible change at the other (paramutable) allele[17]. Paramutation has been described only in plants, and has been especially well characterized at certain anthocyanin loci in maize and snapdragon[17,18]. In many cases, paramutation produces erratic, non-clonal patterns of gene expression, not unlike those associated with co-suppression. An important, defining feature of paramutation is the temporary persistence of the effect on the paramutable allele after the paramutagenic allele segregates away. This effect has not yet been observed in co-suppression. Persistence of methylation following segregation has been observed in pre-meiotic inactivation in fungi[13–15].

Epimutation is a heritable change in gene function due to modifications, such as DNA methylation, which do not alter nucleotide sequence[19]. The stability of epimutation fluctuates with unstable epimutations giving rise to stable epimutations, and vice versa. Epimutation has been well characterized in studies of activity cycles in three different transposable element systems in maize[9,20–23]. The *Suppressor-mutator (Spm)* transposable element has been shown to *trans*-activate and extensively demethylate an inactive homologous element in a manner that depends on the expression of *Spm*[24,25]. Fedoroff and co-workers[9,24,26] proposed that the cycling of activity of *Spm* is determined by an *Spm*-encoded autoregulatory transcription factor whose action depends on the methylation state of the target element and that activation influences further methylation or demethylation of the element.

The likelihood that co-suppression is a homology-dependent process suggests that it could be useful to consider paramutation and epimutation from this new perspective. Since transposable elements (TEs) are usually present in multiple copies, cycling of TE activity may, in part, be due to a homology-dependent interaction between two members of a transposon family. Such an interaction could be an alternative to a transcription-factor-mediated mechanism for cycling of TE activity or an integral part of such a mechanism. Hypotheses invoking such a mechanism would predict that the sequence context or location of a TE would be a determinant of the frequency and developmental control of its changes in activity.

The activity of petunia CHS gene pairs that are able to participate in co-suppression events is under developmental control, similar to that affecting the activity of *Spm*. Fedoroff *et al.*[9] have suggested that the relationship between methylation state and *Spm* expression is sufficiently complex to warrant being considered a possible mechanism for developmental determination and differentiation in plants. Transgenes which undergo cycles of epimutation could be useful for investigating the basis of epigenetic variation[6]. Pigment transgenes may be particularly useful for such studies since they permit the direct visualization of gene expression on a cell-by-cell basis in living tissue[1,2,8]: the observation of abrupt, co-operative changes in pigment gene activation in adjacent cell layers of a new branch[6] (Box 1) suggests that

this system may provide a convenient model for the study of the processes of cellular differentiation in plants.

### *trans* interactions in animals

To our knowledge, there are no reports of phenomena resembling co-suppression in animals. The best-known *trans* interaction in animals is the phenomenon of transvection in *Drosophila*[26]. Transvection is an interallelic interaction that differs significantly from co-suppression and paramutation in that it does not occur when the alleles are in different places in the genome, but only when they are at homologous positions on the same chromosome. Transvection is perturbed by mutations of a DNA-binding transcription factor, the *zeste* protein[27], suggesting that the *trans* interaction in transvection is mediated by the transcription complexes of the alleles involved. As in paramutation, only certain combinations of alleles participate in transvection. Interestingly, all the alleles that are known to participate in transvection and have been molecularly characterized are sequence re-arrangements, such as transposon insertions, deletions and inversions that might have significant effects on sequence context. It could therefore be worth considering whether altered sequence context is a determinant of transvection, as well as whether co-suppression involves interactions between transcription complexes.

### Summary

We have attempted to explain co-suppression in terms of a hypothesis whereby homologous sequences are able to interact somatically *in trans*, in a manner influenced by sequence context or location. We have speculated that there might be mechanistic similarities between co-suppression and some other *trans* interaction and epigenetic phenomena in plants, fungi and animals; and that it might be the sequence context in which a gene lies, or its location in the genome that influences the likelihood that it will participate in these phenomena. We suspect that elucidation of the mechanisms behind these phenomena will play a role in developing a better understanding of the relationship between nuclear architecture and gene expression. This, in turn, will be helpful in understanding the developmental regulatory mechanisms that exert control over somatic *trans* interactions in plants, and perhaps in understanding some aspects of the basis of cellular differentiation in development.

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I greatly appreciate the many stimulating discussions and useful insights that contributed to the ideas presented here, especially those with Dick Flavell, Tim Robbins, Eric Selker, Carolyn Napoli, Hugo Dooner, Enrico Coen, Marjori Matzke, Nina Fedoroff, Tom Gerats, and Jos Mol and his group. I also appreciate the permission to mention unpublished results from Shaun Hobbs, Steve Rothstein, Ortrun Mittelsten Scheid, Ry Meeks-Wagner, Don Grierson and Peter Meyer.

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# Developmental Regulation of Co-suppression In *Petunia hybrida*

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## 1 Introduction

The gene silencing phenomenon to be discussed here, initially termed "co-suppression" (NAPOLI et al. 1990; VAN DER KROL et al. 1990; JORGENSEN 1990), was observed in purple-flowered petunia plants genetically modified by the introduction of DNA containing a chalcone synthase coding sequence under the control of the strong CaMV 35S promoter and the 3' end from the nopaline synthase gene of *Agrobacterium*. The selectable marker gene consisting of the coding sequence for neomycin phosphotransferase under the control of nopaline synthase promoter and with the 3' end from the octopine synthase gene was also inserted on the same T-DNA. These genes were introduced into petunia cells via the transferred portion of the Ti plasmid of *Agrobacterium tumefaciens* (i.e. the T-DNA).

Chalcone synthase is a key enzyme in flavonoid biosynthesis and, therefore, in pigment production. These pigments are synthesized intensely in the epidermis of flower petals, but also to lesser extents in many other parts of the plant including the anthers. Pigment production is cell-type specific. Chalcone synthase gene expression is transcriptionally regulated but separate post-transcriptional effects have been described that influence the pigmentation pattern in flowers (MOL et al. 1983). In petunia, chalcone synthases are encoded by a gene family (KOES et al. 1989), and the cDNA used to create the new transgene was

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from the chalcone synthase A (CHS A) allele responsible for most of the chalcone synthase activity in petals (Koes et al. 1989). Surprising phenotypes were produced in that a very high proportion of the first family of primary transformants had flowers with white sectors, and the flowers of some plants were completely white (Napoli et al. 1990). This was interpreted to imply that the introduction of a new chalcone synthase gene had caused the loss of most or all chalcone synthase activity from the inserted transgene and the endogenous chalcone synthase genes in the white petal sectors. This interpretation was confirmed by the correlation between the lack of anthocyanin pigment and the very low levels of mRNA from both transgene and endogenous CHS A in petals (Napoli et al. 1990; Van der Krol et al. 1990). This suppression of both kinds of homologous gene was the reason for using the term co-suppression to describe the phenomenon (Napoli et al. 1990). Subsequent analyses of large numbers of transformants and their progeny from the selfing and backcrossing of selected transgenic lines have revealed numerous important features about the co-suppression phenomenon (Jorgensen 1993b, 1994, and unpublished results).

## 2 Co-suppression and Plant Development

The flower phenotypes showing co-suppression have been classified on the basis of the position and extent of pigmentation in the flowers (Jorgensen 1993a,b). This classification is meaningful because phenotypes are characteristic for particular transformants even though new variants may arise, as described below. Some of the phenotypes are shown in Fig 1. They range from completely white where pigment production is suppressed in all parts of the flower—tube, corolla and anthers—to other patterns where the white segments are small. In one pattern the pigmentless sector is confined to the tube and the anthers, but frequently extends just outside the tube and to a greater extent on the lower petals. In others pigment loss occurs in small sectors along the veins and/or petal tips. In another pattern, pigment loss is orientated along the edges of the petals. The areas without pigment can be much larger in some phenotypes (Napoli et al. 1990). In yet another series of plants, the white sectors are small and dispersed across the flower in complex patterns. All these patterns point to inherent features of flower development that are revealed by the transgene. The cells which lack pigment are not simply clonally related. Instead it appears that cells occupying certain locations in the floral meristem with respect to architectural features of petal shape such as lines of symmetry, respond similarly (but not identically) from petal to petal to the presence of the transgene, and these responses are different from those of other cells in other positions. The pattern boundaries are coincident in the upper and lower epidermis. Thus pattern formation may also require intercellular communication.

The untransformed parent plants show no evidence of such pigmentation patterns, though other varieties do (Red Star and Velvet Picotee; Mol et al. 1983;

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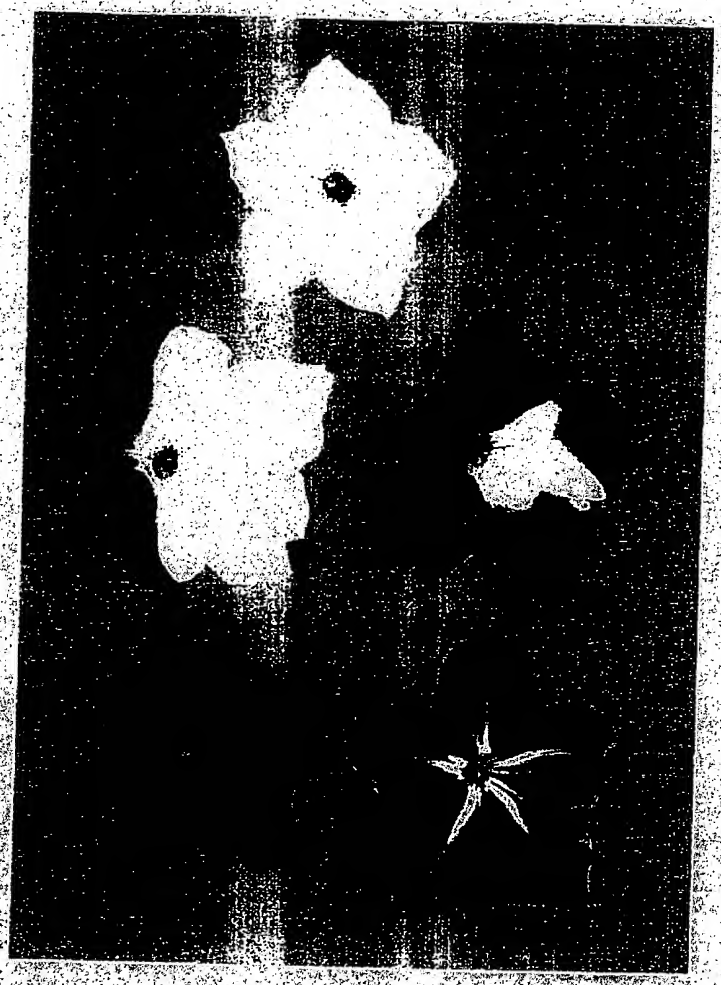


**Fig. 1.** Flower patterns resulting from insertion of the transgene consisting of the coding sequence of CHS A under the control of the CaMV 35S promoter (see colour version of the figure on the backcover). The untransformed parent has only purple flowers. Phenotypes from top middle round to bottom left display: extensive co-suppression emanating from lower petal junctions, complete co-suppression, co-suppression from lower petal junctions, co-suppression along petal veins.

Van Blokland 1994). From the principles of flower design, one can assume that the architectural basis of the patterns is not caused by the transgene but is an inherent feature of flower development. However, elements of this feature somehow interact with the transgene or its product to produce the observed patterns, and different versions (states) of the transgene interact differently to create the different patterns (Jorgensen 1993a,b).

The observation that many transgenic plants display a characteristically patterned flower phenotype, based on the patterns of co-suppression, implies that the 'state' of the transgene is somatically inherited. When the meiotic inheritance of transgene effects on flower phenotypes was examined (Jorgensen 1993b and unpublished), several outcomes were noted. In many cases, the phenotype bred true and is thus germinally stable. In other plants examined, a new range of somatically inherited phenotypes was observed. For example, from

a backcross between a white transformant containing two tandem copies of the new genes and its untransformed parent, many phenotypes were obtained including fully purple, fully white and various patterned types. In these cases the phenotype based on the floral positions of cells showing co-suppression is germinally unstable and the transgene presumably alters its state. Thus it can be concluded that a given transgene can exist in different epiallelic states, and these states can change during meiosis or early embryonic development (JORGENSEN 1993b). Occasionally a lateral branch emerges that displays a different flower phenotype with more or less pigmentation, and the variation is inherited, implying that a change has occurred in the L2 layer of cells in the flowers (JORGENSEN 1994 and personal communication).

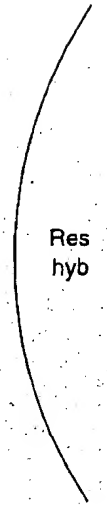
Petunias produce flowering branches from organised groups of cells (meristems) in the axils of leaves or on the flanks of meristems. A genetically different branch results if the group of cells in the meristem flank becomes modified. Occasionally single variant flowers, gradients of phenotypic change as a branch ages, and simultaneous changes in different branches have been noted (JORGENSEN 1994) implying that changes can occur in any floral meristem. Because the inherited L2 layer and the L1 layer in which epidermal pigment is produced are separate developmental lineages of cells, it is reasonable to consider the possibility that the changes in transgene state behind pattern changes occur in many cells of a meristem essentially simultaneously.

The remainder of this chapter deals with the origins of the pigmentless phenotype created by the insertion of the CHS A coding sequence under the control of the CaMV 35S promoter.

## 3 Hypotheses to Explain Gene Silencing

Numerous examples are known, in at least six plant species, where gene inactivations results from the introduction of additional homologous sequences. These have been reviewed elsewhere (JORGENSEN 1990, 1991, 1992; MOL et al. 1991; KOOTER and MOL 1993; MATZKE and MATZKE 1993; MATZKE et al. 1993; ASSAAD et al. 1993; VAUCHERET 1993; GORING et al. 1991; MEYER et al. 1993; GRIERSON et al. 1991; FLAVELL 1994; HART et al. 1992; MEINS 1989; MEINS and KUNZ 1994), and in other chapters in this book (for example see Hamilton et al. and de Lange et al., this volume). They will not therefore be discussed extensively here. However, it should be noted that no single mechanism can explain the variety of examples where loss of gene expression has occurred.

Four kinds of hypotheses have been put forward to explain the diversity of gene silencing phenomena. In the first, inactivation of transcription is postulated due to the physical interaction (ectopic pairing) in the nucleus of the duplicated but non-allelic sequences (loci). Cycles of DNA–DNA or chromatin–chromatin interactions (see Fig. 2) could leave the chromatin structure or methylation patterns of the participating genes in different states which could consequently interfere with

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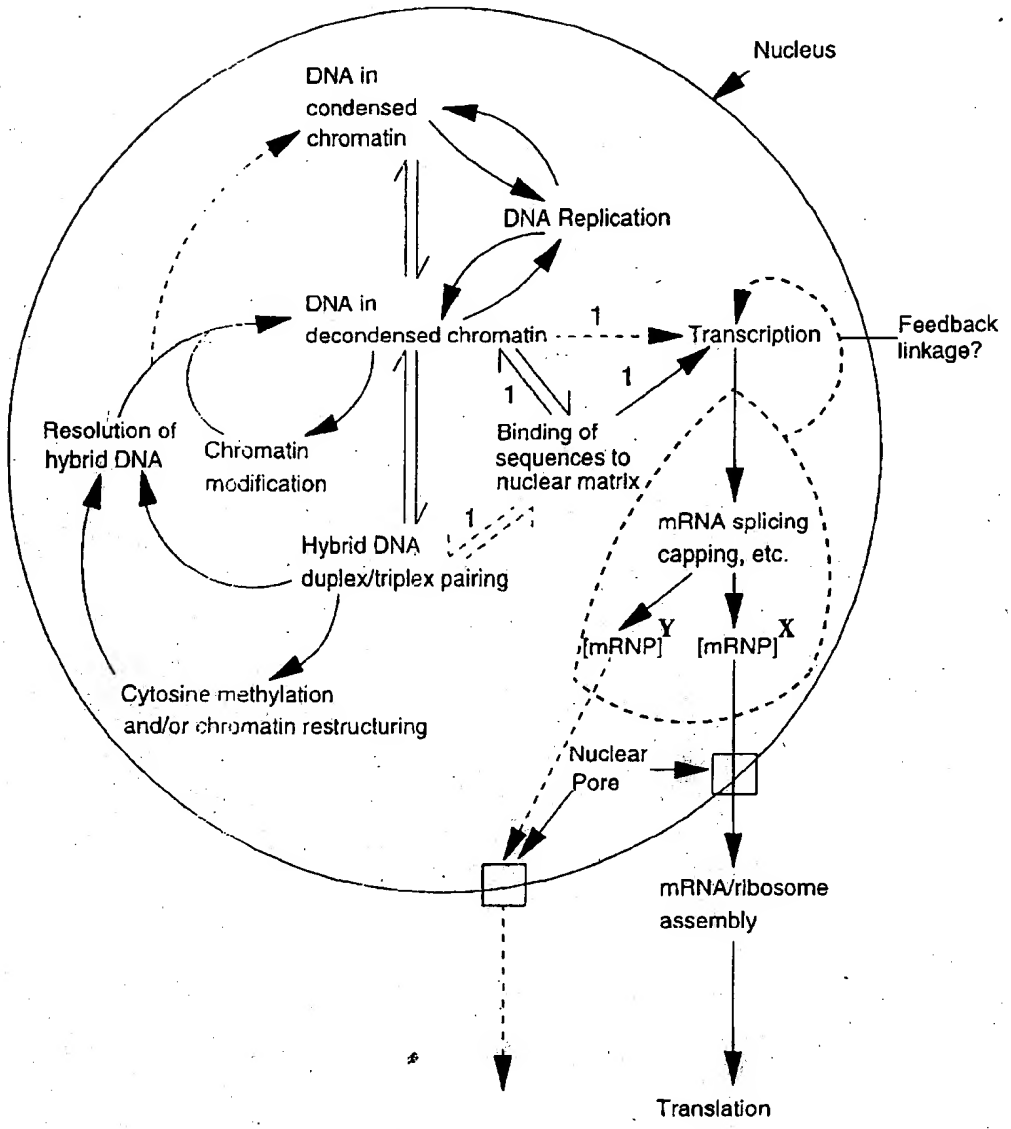


**Fig. 2.** Cellular processes relevant to models for gene silencing. The network of pre-transcriptional events illustrates how the structure of decondensed chromatin, the substrate for transcription, can be modified by various sorts of changes including cytosine methylation and interactions with homologues including transgenes. The changes could modify decondensed chromatin such that it does not bind properly to the nuclear matrix or bind transcription complexes efficiently. After "normal" transcription mRNA is processed, capped and polydenylated in messenger RNA nuclear protein particles $[mRNP]^X$ which are then exported from the nucleus and the mRNA traslated on ribosomes. Where gene silencing is post-transcriptional, transgene mRNA processing, splicing, capping or polyadenylation could be aberrant, thereby leading to synthesis of aberrant particles $[mRNP]^Y$. These might not be translated efficiently and may be substrates for RNases. They may also be substrates for antisense RNA formation. In any event they do not give rise to protein product. There is the possibility of aberrant mRNA production influencing transcription. Further details are described in the text

the assembly of essential transcription complexes or the binding of the chromatin to the nuclear matrix. These processes are labelled 1 in Fig. 2. There is no direct evidence for such interactions occurring in plants, but precedents come from studies on fungi and *Drosophila*. In *Neurospora* and *Ascobolus*, DNA homology-searching processes and hybrid DNA formation have been inferred from the inactivation of duplicated sequences via cytosine methylation in premeiotic cells (SELKER 1990; Foss and SELKER 1991; RHOUNIM et al. 1992; FAUGERON et al. 1990). In yeast, equivalent frequencies of allelic and ectopic meiotic recombination have also been taken to imply the existence of efficient, generalised, DNA sequence homology searching processes (HABER et al. 1991). In *Drosophila* there are many examples where expression of a gene is influenced by "sensing" the presence of another specific gene after some kind of localised somatic chromosome pairing. The pairing could be mediated via DNA, RNA or transcription complexes. The consequentially altered chromatin, sometimes heterochromatic, state created following the interlocus interactions can be clonally inherited when not disturbed by other events (TARTOF and HENIKOFF 1991; HENIKOFF 1992; PIROTTA 1990; WU 1993; PARO 1990).

The second hypothesis is based upon elevated competition between the increased number of genes for non-diffusible sequence-specific factors essential for ordered transcription or translation.

The third hypothesis focuses on post-transcriptional events. It postulates the degradation of the specific mRNAs due to the synthesis of homologous antisense RNAs in the cell, formation of double-stranded RNAs between the antisense RNA and mRNAs and recognition of the aberrant duplexes as substrates for a RNase. Mutual inactivation of homologous mRNAs can often be achieved by the introduction of antisense gene. Double-stranded RNAs may also inhibit translation if they are formed in the cytoplasm (TEMPLE et al. 1993; CORNELISSEN and VANDEWIELE 1989). Evidence for the existence of dsRNA in plants is however very weak (GRIERSON et al. 1991; JORGENSEN 1991; MOL et al. 1991). These antisense RNAs could be made from an unknown promoter close to the transgene functioning in the appropriate orientation, possibly by readthrough from a neighbouring gene or by the action of RNA-dependent RNA polymerase on aberrantly accumulated mRNAs (LINDBO et al. 1993; FLAVELL 1994). This latter enzyme exists in plant cells.

The fourth hypothesis postulates the inhibition of transcription and/or translation by feedback from a specific gene product that accumulates in aberrantly high concentrations in the transgenic plants. This would constitute a self-induced, autoregulated control system (HART et al. 1992; MEINS 1989; MEINS and KUNZ 1994).

## 4 Mechanisms and Hypotheses for Co-suppression of Chalcone Synthase in Transgenic Petunias

We now consider the petunia chalcone synthase case in the light of these hypotheses. Throughout this discussion, it is relevant to bear in mind that not all transgenic plants containing CHS A transgenes display co-suppression in the petals, and that in some plants only specific segments of petals show co-suppression. Also, it is important to remember that inherited somatic and meiotic changes can occur to influence the extent to which co-suppression is observed.

In the process of making the transgenic plants, it can be expected that different numbers of T-DNAs become stably inserted into different petunia plants and display different structures. Tandem arrays of T-DNAs are common, as are copies inverted with respect to one another. Genetically unlinked T-DNAs also accumulate. Thus plants with different numbers of active genes are likely to be produced, as noted in other studies on transgenic plants (Hobbs et al. 1993; Assaad et al. 1993, Scheid et al. 1991; Linn et al. 1990). It will be important to investigate thoroughly whether the structure of T-DNA inserts influences the extent of co-suppression and the kinds of flower pattern produced. Van Blokland (1994) has concluded that phenotypic effects of CHS A transgenes are correlated with the presence of inverted repeats of T-DNA.

Where there are multiple copies of the chalcone synthase transgene then the copies might interact (see Fig. 2) to silence transcription of one another and the endogenous CHS A genes. Such silencing has been recorded for several sorts of transgenes (Peach and Velten 1991; Assaad et al. 1993; Hobbs et al. 1990, 1993; Elkind et al. 1990; Linn et al. 1990; Matzke et al. 1994b; Vaucheret 1993). Inverted repeats seem to be more frequently associated with transcriptional silencing (Hobbs et al. 1993) How such physical interactions occur is unknown, but they may be the means whereby one or more of the duplicated sequences gain some methylated cytosines. No evidence for silenced CHS A genes becoming routinely methylated has yet been obtained in investigations of several sites within the coding sequences and promoters.

There is evidence, however, that in some transgenic petunias, CHS A transcription is not blocked in petal cells showing loss of pigment. Run-on transcription assays on nuclei from isolated purple and white petal sectors from the same plant show similar levels of CHS A transgene and endogenous CHS A transcription (Van Blokland 1994). Furthermore, similar levels of unprocessed nuclear endogenous CHS A transcripts have been detected in flowers of some co-suppressed and non co-suppressed variant plants in our laboratory and in that of Mol and co-workers. The levels of RNA transcribed in isolated petal nuclei are not correlated with the extent of chalcone synthase suppression (Van Blokland 1994; Kooter and Mol 1993; Mol et al. 1991). These details are reviewed in another chapter in this book (de Lange et al., this volume). We have also found in some plants that white flower sectors retain high levels of CHS A RNA, making it likely that post-transcriptional losses of functional mRNAs are the cause of or a

major contributor to the co-suppression phenotype. Studies of inactivation of some other transgenes in plants have also concluded that the inactivation is post-transcriptional (Smith et al. 1990a; de Carvalho et al. 1992; Bate et al. 1992; Meins and Kunz 1994).

In plants where transcription of the CHS A transgenes is not blocked but steady state functional mRNA levels are very low, then a major cause of co-suppression could be accumulation of excess levels of antisense RNAs to the CHS A mRNA, double-stranded RNA formation and degradation of the duplex RNA (third hypothesis above). The presence of antisense RNA to chalcone synthase has been investigated in transgenic petal tissues differing in co-suppression, i.e. purple and white. Of the particular variants studied by us, most were derived from the same transgenic parent and possess two copies of the transgene in inverted orientation. Reverse transcriptase and primers specific for antisense RNA were used to make DNA copies of RNA in RNA extracts isolated from white or purple sectors. Antisense chalcone synthase RNAs were found in both white and purple flower sectors but only in transgenic plants. It is, therefore, concluded that the antisense RNAs are due to the transgene. The finding that antisense chalcone synthase RNAs are in both white and purple sectors suggest that if antisense RNA is essential for the loss of mRNA and gene expression in this genotype, it is clearly insufficient. Similar conclusions have been drawn by Mol and co-workers (de Lange et al, this volume; Van Blokland 1994) who used other assays to detect antisense RNA.

How is antisense RNA produced from the transgene, what is its structure, and how does it function? These important questions still have to be examined experimentally. It will be necessary to examine many different transgenic plants with different numbers and kinds of transgene structures since it is not clear how antisense RNA could be produced so efficiently in all transformants (Jorgensen 1991). Where antisense RNA is not transcribed from defined genomic promoters it could be formed by an RNA-dependent RNA polymerase using sense mRNA as template (Lindbo et al. 1993; Flavell 1994).

On simple considerations of how antisense RNA interferes with sense mRNA, it would be assumed that the higher the antisense to sense RNA ratio, the more efficient would be the loss of sense gene expression. Some data in plants to support this have been produced (Smith et al. 1990b; Hamilton et al. 1990; Cannon et al. 1990; Robert et al. 1989; Van der Meer et al. 1992). However, there are many reports of discrepancies between the relative levels of antisense RNA transcripts and loss of sense gene expression (reviewed in de Lange, this volume; Cannon et al. 1990; Stockhaus et al. 1990; Van der Krol et al. 1988). Van Blokland (1994) found in petunias transgenic for chalcone synthase that antisense transcription could be high in the absence of co-suppression or vice versa. If antisense RNA is the cause of degradation of CHS A mRNAs, but overall steady state or transcription levels of antisense mRNA do not correlate with co-suppression it must be a small fraction of the antisense RNA that is critical, and this fraction must have efficient access to the unprocessed primary RNA transcripts or mRNAs formed after processing, capping and poly-A tail additions. This implies

that the variation in co-suppression in transgenic plants that makes antisense and sense RNAs could involve variation in the accessibility of antisense and sense RNAs to each other in the nucleus or the cytoplasm (see later).

Post-transcriptional loss of CHS A gene expression and pigment production could, alternatively, be due to the accumulation of excess levels of CHS mRNA and the consequential induction of an mRNA-specific process, that is able to catalyse the inactivation and/or degradation of transgene and endogenous CHS A mRNAs (fourth hypothesis above). This hypothesis includes the notion of critical localised threshold levels of mRNA in a cell. Where mRNA levels are below the threshold, purple pigment is produced; in contrast, when the level is exceeded, active mRNA is lost and no pigment is produced. There are several predictions from this model.

1. In plants where all flower epidermal cells lack pigment, the threshold mRNA concentrations are likely to have been exceeded in all cells of the plant. This is as observed in many white flowered plants where levels of transgene mRNA are very low in stems and leaves, as well as flowers (Napoli et al. 1990; this laboratory, unpublished).
2. In plants which have purple flowers with white sectors, the levels of localised active mRNA is likely to be higher than that found in plants that make only purple flowers. This is because in such plants small increases in the level of mRNA accumulated would exceed the threshold more readily and thus lead to the pigment loss. Some evidence has been gained to support this hypothesis in that the levels of transgene CHS mRNA accumulating in leaves of transgenic plants with purple and white flowers is greater than in leaves of transgenic plants forming only purple flowers (unpublished results).

If localised mRNA concentrations are the determinant of the post-transcriptional trigger for co-suppression, then the critical parameters affecting co-suppression would be the rate of transgene transcription and/or changes in the efficiency (rate) of mRNA transport through the nucleus, of export through the nuclear envelope, of binding to the ribosome and of translation. Changes in flower pigment production due to different levels of translatable CHS A mRNA could therefore come about through (a) pre-transcriptional events including changes in the levels of transcription factors, restructuring of chromatin, (and/or) changed cytosine methylation to affect the affinity of the transgenes for transcription complexes as noted earlier (see Fig. 2), and/or (b) changes in the rate of mRNA transport, etc. The latter could result from the transgene altering its position in the nucleus with respect to nuclear transport channels and the supply of protein components of the mRNPs essential for correct mRNA processing, transport and export (Flavell 1994). Furthermore, aberrantly high levels of mRNA in the nucleus might lead to mRNP particles with a different complement of proteins (Wolffe 1994) from those formed when the CHS genes are optimally transcribed for mRNA processing and transport and when nuclear mRNA levels are much lower (see Fig. 2). Such modified mRNPs might not make the mRNAs available for translation.

A growing number of proteins are known that bind to mRNA and prevent translation as part of specific regulatory mechanisms. The studies on the ubiquitous Y box proteins and the FRG Y proteins of Xenopus oocytes, in particular, are interesting (Wolffe 1994). These proteins bind to mRNAs and inhibit their translation (Bouvet and Wolffe 1994). They will bind to a range of double and single stranded DNAs and RNAs including specific Y box sequences in gene promoters (reviewed in Wolffe 1994). They also stimulate mRNA synthesis, but not necessarily by binding to the promoter motifs. FRG Y2 (a predominantly nuclear Y box protein) has been immunolocalised to nascent transcripts on lampbrush chromosomes (Sommerville et al. 1993). These observations are consistent with a direct role for FRG Y2 in packaging mRNA in the nucleus and for somehow linking the competence of the mRNA for translation with its transcription (Bouvet and Wolffe 1994). Recruitment of some other heterogeneous nuclear (hn) RNP proteins on to pre-mRNA is also dependent on transcription (Pinal-Roma and Dreyfuss 1992), and there is a growing list of eukaryotic proteins having dual roles in the transcription and translation processes (reviewed in Bouvet and Wolffe 1994).

If protein-based regulatory systems linking transcription, mRNA packaging and mRNA translatability are present in plants, then they might be responsible for post-transcriptional loss of gene expression following "aberrant" mRNA synthesis from the CHS A transgenes. It is also possible that they could lead to shut down of transcription. This whole area of nuclear biology needs to be explored in the context of understanding how aberrant active CHS A transgenes can promote loss of pigment production in flower petals. While it is possible to imagine how mRNA from an aberrantly located transgene might be sequestered into an inactive mRNP structure, how would this affect mRNAs from the endogenous CHS A genes? Perhaps the protein-mediated regulatory systems could also provoke cross-talk between homologous mRNP complexes and sequester all CHS A RNAs into aberrant mRNPs.

If excess CHS mRNAs accumulate due to higher rates of transcription or low rates of mRNP maturation in the nucleus and/or translation, how are they degraded? This could result from the aberrant mRNPs being recognised by RNases and the RNAs consequently degraded (Sachs 1993; Sullivan and Green 1993). Alternatively, antisense RNA could be produced on the accumulated mRNA templates by RNA-dependent RNA polymerase, and these double-stranded RNA structures are subsequently degraded. Cycles of RNA production of both antisense and sense RNAs could emerge from this process to provide an autocatalytic system for the production ds RNAs. Any such antisense RNAs could, of course, explain loss of both transgene and endogenous CHS A mRNAs.

## 5 Concluding Perspective

From surveying the range of examples of gene silencing, it is clear that multiple mechanisms contribute to the observed phenotypes and in some examples

mechanisms resulting in inhibition of transcription are major determinants, while in others post-transcriptional events occur. This diversity of mechanism may also appear between plants genetically altered by insertion of the same or related transgenes, but at different sites, in different arrangements and with different effective promoter strengths. Thus it is desirable not to automatically lump all petunias involving CHS A transgenes into a homogeneous group and attempt to find a single mechanism for the observed gene silencing or lack of it.

The scenario described above for the post-transcriptional control of chalcone synthase silencing may also be combined with, or lead to, variable patterns of transcription silencing in different genotypes. The data accumulated to date point to association of the phenomena with higher levels of mRNA synthesis and/or antisense RNA, and it has been argued that there are probably more than one cellular pool or RNP package for each of these molecules. The fact that sense mRNAs, may exist in different RNP pools and packages implies that we need to look for the different structural forms that may have different stabilities and opportunities to associate with ribosomes and be translated. Similarly we need to investigate whether different antisense RNPs exist.

If only one of the antisense RNP pools is available to interact with only one of the classes of sense mRNP, then the interacting classes are likely to be degraded in co-suppressed tissues, while other classes or pools might not be. Such discoveries might help explain the lack of correlation between antisense RNA to sense mRNA ratios and co-suppression phenotypes in different plants and tissues.

Hypotheses that propose the formation of different mRNP packages from active transgenes in aberrant nuclear positions and the production of pools of antisense RNA in some cases offer the following sorts of explanations for the origins of purple and white flower sectors: In transgenic plants where CHS A transgene transcripts are efficiently processed, packaged and exported then aberrant mRNPs would not accumulate and so co-suppression would not occur. Such plants would have purple flowers. If antisense RNPs were produced in such plants, the antisense RNA might not be accessible to the sense mRNPs and so the flowers would be purple. If, however, transgene mRNA were processed, packaged and exported inefficiently, due to the location or other features of transgene chromatin, then critical levels of nuclear mRNA would be exceeded, packaging could be aberrant and a different mRNP structure for all CHS A mRNAs might result. Messenger RNA in this structure might not be translated, or might be accessible to RNases and antisense RNAs or to RNA-dependent RNA polymerase that makes antisense RNA. Any of these would result in the formation of white flowers.

These ideas are testable and imply that switches in pigment production during transgenic CHS A plant development could result from (a) a change in nuclear position of the transgene; (b) a change in transcription rates (these would constitute inherited changes in state of a transgene); (c) a change in cell physiology influencing nuclear processing, RNA packaging, export and mRNA translation rates; and/or (d) a change in antisense RNA synthesis. Variation in such parameters would not be surprising during meristem development, growth in

different environments or during specific developmental phases. The patterns in flowers are presumably due to similar changes.

The developmental changes influencing the nuclear metabolism of specific clusters of cells in floral meristems and floral tissues are unknown. However, analyses of the CaMV 35S promoter have revealed that it contains multiple elements that respond differently in different petunia floral tissues, leading to differential transcription (BENFEY and CHUA 1989; BENFEY et al. 1989). Thus the enhanced probability that co-suppression occurs in the flower tube and veins in some transgenic genotypes containing CHS A under the control of the CaMV 35S promoter or in other regions in other genotypes could be due to differential interactions between the promoter and enhancer structures of the promoter and the transcription factor concentrations present in different sectors of the meristem and floral tissues.

In conclusion, it is clear that studies into the origins of co-suppression and gene silencing in general will teach us many new features of cell biology and the control of gene expression. Furthermore, because of the wish to create agriculturally novel transgenic plants, understanding how active transgenes can lead to gene silencing is of considerable commercial interest.

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# Gene silencing: **Cosuppr ssion at a distanc**

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**When a plant carries a transgenic copy of an endogenous gene, both genes may be silenced. This 'cosuppression' can occur not only within individual cells, but also in distant cells through an agent that apparently moves through the plant's phloem.**

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Individual genes are normally active in patterns that are well defined in time and place within an organism. Occasionally, however, a gene is expressed (or inactivated) in a sporadic and unpredictable manner. These 'epigenetic' events may be transmitted to daughter cells, but they do not persist indefinitely. In this way, they differ from mutations — permanent inherited changes in the genetic material.

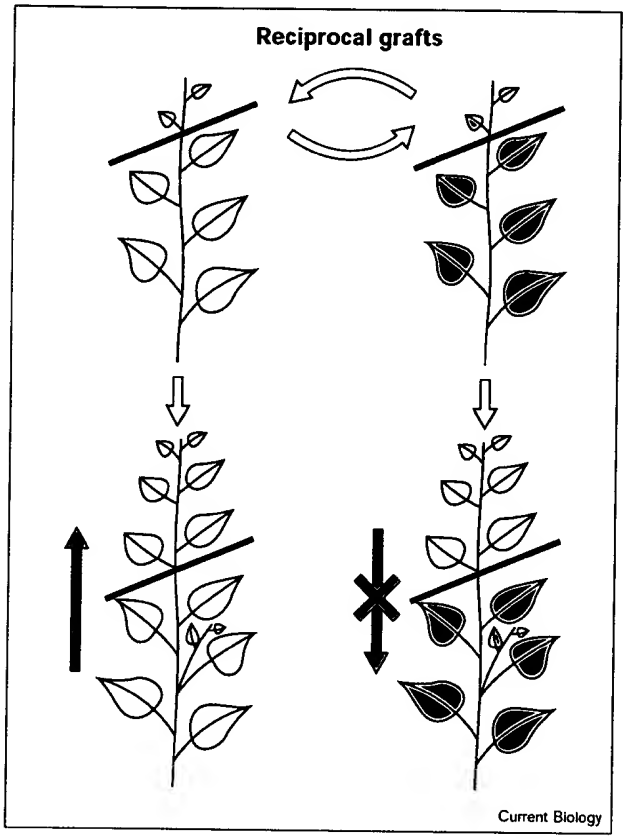
One epigenetic phenomenon becoming a focus of great interest is gene silencing. Here, a gene is inactivated in cells where it is normally functional. In plants, one type of gene silencing is dependent upon the abnormal presence of repeated copies of the gene [1,2]. It seems that the extra gene copy can sometimes become inactivated, but, surprisingly, so does the normal endogenous gene. This joint silencing was called cosuppression, and was brought to light seven years ago, when petunias were transformed with genes encoding biosynthetic enzymes in the anthocyanin pigment pathway [3,4]. Cosuppressed petal cells showed up as white, pigment-free patches or zones in an otherwise purple background. Many other examples of cosuppression have been reported subsequently, as plant scientists realised that variable and varying inactivity of inserted transgenes is a common phenomenon.

The mechanisms of cosuppression have been intensively investigated in plants [1,2], not least because economic outcomes can depend upon silencing not occurring. From the available data, a number of important generalisations can be made, including the following. First, many cases seem to involve the inactivation of RNA transcripts (post-transcriptional silencing) of both the transgene and the endogenous gene. Second, some DNA sequence homology between the transgene and the endogenous gene is necessary, but neither need encode a functional protein product. Third, in many cases it has been shown that the genes must be actively transcribed before cosuppression can occur. And fourth, the frequency of cosuppression, and its reversal, is very variable, but the rate is generally proportional to the number of copies of genes present, and to their rate of transcription. Many of these findings have implicated RNA as an agent in cosuppression (see [5], for example). Furthermore, the idea that cosuppression involves a switching process, reinforced by positive feedback, has experimental support. (It should be pointed out that, as originally defined, 'cosuppression' refers to post-transcriptional silencing alone, and only to cases where all copies are silenced. The more general terms 'repeat-induced gene silencing' or 'homology-dependent gene silencing' were later introduced to cover a wider range of phenomena where multiple copies of a sequence lead to silencing, irrespective of whether this occured before or after transcription, and whether or not all the sequences were silenced [1].)

A clever series of grafting experiments has recently thrown light on the mechanism of cosuppression [6]. The experiments tested whether silencing can be induced at sites distant from a silenced zone within the plant. Tobacco plants were available that had been transformed with a gene, *Nia2*, encoding nitrate reductase under the control of the strong 35S promoter of cauliflower mosaic virus. In a proportion of these plants, the 35S:*Nia2* transgene and the endogenous *Nia2* gene were cosuppressed, causing yellowish, sickly growth. Reciprocal grafts of growing shoot tips were made between active and silenced plants of this transgenic strain (Figure 1). The intriguing finding was that shoots from non-suppressed transformed plants rapidly became cosuppressed as they grew on cosuppressed stocks. (As expected, the cosuppressed tips continued to exhibit cosuppression as they grew on normal stocks.) It could, of course, be argued that the sickly growth of the stock induced silencing in the grafted shoot, but this possibility was eliminated by grafting normal transgenic tips onto *nia2* mutant plants that exhibit the same sickly phenotype, but as a consequence of mutation, not cosuppression: in this case the grafted shoots remained green and non-suppressed as they grew.

One possible explanation for these observations is that an agent moves upward from the cosuppressed stock into the new grafted shoot, rapidly cosuppressing its *Nia2* genes. The agent does not seem to move the other way, as new side shoots growing from non-suppressed stocks that host grafted cosuppressed shoots remained non-suppressed (Figure 1). Additional grafting experiments [6] showed that, whatever it is that moves into and converts the newly growing tip, it arises from the leaves and/or stem of the

**Figure 1**



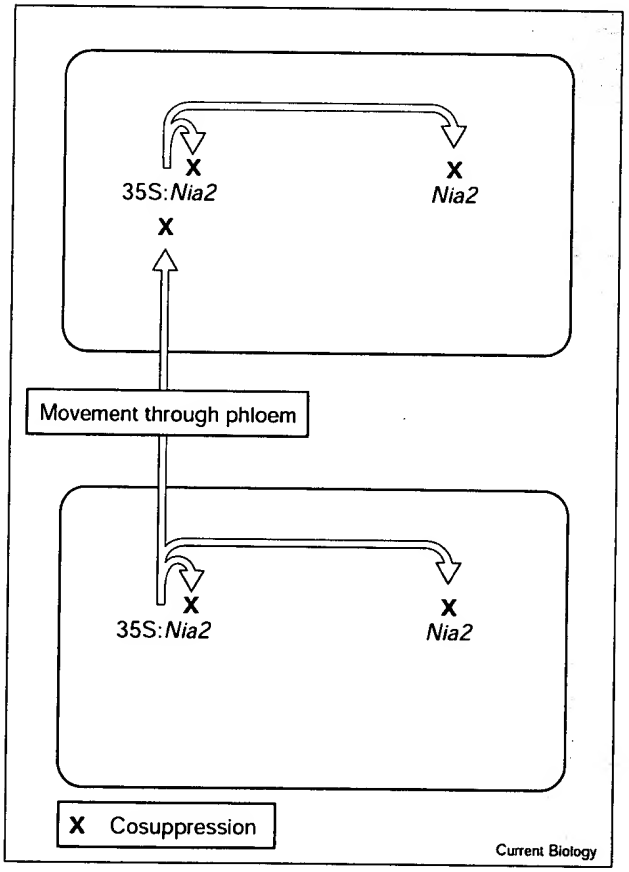

Results of reciprocal grafts between silenced (yellow) and unsilenced (green) forms of a tobacco line carrying a transgenic nitrate reductase gene (35S:*Nia2*). Cosuppression of both the transgene and the endogenous *Nia2* gene is induced in the unsilenced shoot when grafted onto the silenced stock (left, red arrow). In the reciprocal grafted plant, however, the silenced shoot continues to be silenced on the active stock, but the latter remains unsilenced (right).

cosuppressed stock, not from the roots. It was also shown that the agent could move through at least 300 millimetres of normal wild type stem.

Four further experiments were performed, leading to the following conclusions [6]. First, the cosuppressed stock disrupted expression in grafted shoots only if they were transgenic. If non-transgenic tips were used, expression of the wild type *Nia2* gene was normal. Second, it did not matter where the transgene had inserted in the host genome. Different strains carrying the same transgene were all susceptible to cosuppression. Third, silencing was effective only against the same transgene, other transgenes were unaffected. Fourth, the process was not limited to the nitrate reductase gene. Parallel experiments using the nitrite reductase gene *Nii2* gave similar results. Significantly, a transgenic reporter gene from bacteria could also be silenced from afar. This is important, because this bacterial transgene has no effect on the plant's growth or metabolism, so such factors must be irrelevant for the successful transmission of silencing. Taken together, the observations show that the proposed silencing agent works at a distance, and that it acts through the homologous transgene in the target cells (Figure 2).

What is the nature of the silencing agent? The most likely scenario is that it is an RNA transcript from the transgene, or a derivative of this RNA. There is now considerable evidence that post-transcriptional gene silencing may often be associated with specific degradation of RNA transcripts [1,2,5]. The mechanisms are not known, but indirect evidence in some cases suggests that aberrant transcripts from

**Figure 2**




Interpretation of the genetic basis of systemic acquired silencing. In the silenced stock (below), the silencing agent is apparently derived from a transgene (in this case the 35S:*Nia2* transgene), and it silences itself and the endogenous gene (*Nia2*) post-transcriptionally. The agent moves up the plant into the unsilenced grafted shoot (above), possibly in the form of RNA that moves via the phloem. Here it interacts specifically with the same or a homologous transgene which is then silenced along with the normal version of the gene.

the transgene itself trigger the process. This could involve base pairing with homologous RNA sequences if the trigger sequence had a stretch of complementarity with such sequences. This could occur if the transgene contained inverse repeats, or if RNA-dependent RNA polymerases were active in the cell, for example. If the triggering RNA generates more of itself as a result of the degradation process, cosuppression would be self-perpetuating in cell lineages. Finally, if initiation of the degradation process requires a threshold in the rate of transgene expression, or the rate of formation of aberrant RNA, to be reached, then the stochastic nature of cosuppression could be accounted for. The current observations fit well with these proposals.

The phloem is the likely pathway of movement of the silencing agent. Phloem is a continuous system of vascular cells that acts as a conduit for movement of sugar — the product of photosynthesis — throughout the plant. There is little known precedent for the movement of a naked RNA molecule through phloem. The closest parallel is provided by RNA viroids and RNA viruses that can move systemically in plants, but, in the latter case at least, only in association with specific virus-encoded proteins [7]. The phloem is also the channel for some other agents, including a flowering factor, 'florigen', that moves from the leaves to the shoot apex. (Florigen has not yet been identified and it is tantalising to think that it might be an RNA species.) Phloem also apparently transmits a peptide, systemin, that is protective against protein degrading enzymes produced by pathogens [8] and is the likely pathway of systemic acquired resistance [9]. In systemic acquired resistance, expression of a series of protective genes is triggered at a distance from a site of a pathogen attack. By analogy, Palauqui *et al.* [6] have called the remote induction of cosuppression 'systemic acquired silencing'. Another example of systemic movement of a silencing agent has already been announced [10]: when transgenic *Nicotiana benthamiana* plants were locally infected with *Agrobacterium* carrying another copy of the transgene, the 'endogenous' transgene was silenced in newly developing leaves far distant from the infection site.

Recent experiments have shown that close parallels exist between gene silencing and one form of resistance of plants to viral infection [11]. This was originally discovered when resistance to viruses was unexpectedly obtained in plants containing a transgenic copy of a gene from the same virus. It seems that transcripts of the transgene can interfere with normal production of viral RNA. This also works the other way round, as viral RNA can reduce the level of RNA produced from the transgene. The interplay has now been taken one step further in that inactivation seems to occur even in the absence of any transgene. A recent study [12] explains a phenomenon called 'recovery', in which an infected plant develops symptoms upon initial infection, but soon overcomes the infection and puts on new resistant growth. This seems to be directly associated with degradation of incoming infectious viral RNA, in this case perhaps mediated by aberrant viral RNA sequences persisting from the earlier infection. This is clearly advantageous to the plant, of course, but its advantage to the virus is less clear, unless it allows vertical transmission to seeds of the host [12], with occasional outbreaks allowing horizontal dispersal to other plants.

Is gene silencing of general significance in plants? Although the silencing phenomenon was discovered using experimentally created transgenic plants, arguments can be made for its wider import. As well as reducing the impact of viral infection (see above), the process could also inactivate 'infecting' transposons and retrotransposons. It may also provide a mechanism for editing aberrant RNA transcripts. A recent study of the *SUPERMAN* gene in *Arabidopsis* [13] suggests that gene silencing can sometimes occur spontaneously, thus adding epigenetic diversity to an organism's developmental program. It is also applicable beyond plants. Various forms of repeat-induced gene silencing have been described in filamentous fungi [14], and its extension to the animal world is now at hand [15]. It may well be a universal process.

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